

HHMI REVIEW

Daniel Nathans July 24, 1996.

GENOMIC RESPONSE TO GROWTH FACTORS

PROGRESS REPORT: September, 1991 to July, 1996.

Background:

This project began in 1982 with the objective of understanding how growth factors cause quiescent mammalian cells to enter the cell division cycle. When cultured fibroblastic cell lines are grown to confluence and maintained in a quiescent or non-proliferating state (Go) following the last cell division, the addition of serum or suitable growth factors cause the cells synchronously to enter G1 followed by S phase, G2, mitosis, and cell division. At the time we began this project, this seemed like an ideal system for understanding the biochemical events that mediate the effects of growth factors.

From the beginning our focus was on the presumed changes in gene expression underlying the growth factor response. Once it had been found that genes were sequentially activated by serum or purified growth factors (1), the emphasis of the research was the identification and characterization of induced genes and proteins and their possible roles in determining the cellular response. The experimental approach we and others took to identify genes and proteins was the isolation and analysis of cDNAs derived from mRNAs that appeared at different times after cultured fibroblastic cells were stimulated with serum or purified growth factors (PDGF, FGF) (1-4). By this means genes were identified that were induced prior to DNA synthesis within minutes following cell stimulation (immediate early response genes) or within a few hours (delayed early response genes). Activation of immediate early response genes did not require protein synthesis, (3) whereas activation of delayed early response genes did (5). This pattern of gene activation was consistent with a specific regulated transcriptional cascade or genetic program induced by the growth factor.

Immediate early and delayed early genes each comprised an estimated several dozen genes encoding a diverse set of proteins (4-6). Immediate early proteins included various transcription factors, secreted proteins, membrane proteins, cytoskeletal-matrix proteins, and diverse enzymes (7). Delayed early proteins included metabolic enzymes, extracellular proteases, cytokines, membrane proteins, and non-histone chromosomal proteins (5,7). In addition, in both classes there were many previously undescribed proteins.

The largest class of immediate early response genes encoded transcription factors, including members of the AP1 basic-leucine zipper family (c-Jun, JunB, c-Fos, Fra-1 FosB), the Egr1/Zif268 family of zinc finger proteins (8,9), c-Myc, and Id proteins. In addition, we and others identified another family of proteins, Nup475/TIS II/Tristetraprolin (10-12) and related members with a putative novel metal-binding domain (10). Much of our subsequent research centered on selected immediate early transcription factors or presumed transcription factors: Jun/Fos, Zif268, Id3, and Nup475.

Because of their ubiquity we assumed that Jun/Fos and Zif268 transcription factors in particular are partially responsible for activating delayed early response genes, but our experiments were primarily directed at understanding more general properties of these proteins, e.g., DNA binding properties of Jun

and Jun/Fos dimers (13,14) and Zif268 (15), defining growth factor response elements involved in activation of the Zif268 (16) and JunB genes (17), identifying proteins that functionally interact with Jun and Fos (see below), and structural characterization of the novel metal-binding domain of Nup475 (see below). In addition, we have studied the HMG-I delayed early proteins, which were identified in our cDNA screen, both from the point of view of their DNA-binding properties and the role of immediate early transcription factors in the activation of delayed early genes (see below).

For completeness, I mention that in 1991-'92 neuroscientist Paul Worley spent an internal sabbatical year in my laboratory identifying immediate early genes in the brain (primarily hippocampus) that respond to electrical stimulus; various Hughes Associates from my laboratory collaborated with him at that time and thereafter in identifying and characterizing hippocampal immediate early proteins (18-22).

While our research on growth factor-induced proteins was being pursued, a great deal of progress has been made in understanding transmembrane signaling by growth factors and cytokines, the link between transmembrane signaling and the activation of immediate early genes, regulation of the cell cycle, and the biochemistry of transcription factors. Most directly relevant to our recent and future work is the characterization of latent cytoplasmic transcription factors of the STAT family (Stat 1 through 6 and variants thereof) that are activated by phosphorylation involving cell surface receptors for growth factors or cytokines and the JAK family of tyrosine protein kinases (recently reviewed in 23,24). These will be discussed in more detail below.

Research since September, 1991

Jun/Fos-interacting proteins (25-27) There are three members of the Jun family and four members of the Fos family. These bZip proteins act as Jun homodimers and Jun-Fos heterodimers. We and others have shown that the optimal DNA-binding site sequences for the various dimers are identical or nearly so, corresponding to the highly conserved basic regions of the proteins. Therefore, their differential transcriptional effects are not likely to be due to DNA site specificity, but more likely to specific interaction with other proteins. This was one reason we undertook a screen for Jun- and Fos- interacting proteins. A second reason was to look for other proteins that could dimerize with Jun or Fos in addition to members of the CREB/ATF family already characterized at that time.

Pierre Chevray (then a graduate student) set up the Fields *S. cerevisiae* two hybrid system (28,29) to screen for mammalian proteins that interact with the bZip of either Jun or Fos. With help from Stanley Fields and Philip Hieter, Chevray developed suitable yeast strains and vectors to screen mammalian cDNA libraries routinely. (At the time, this was not a trivial accomplishment, as evidenced by the numerous requests for strains and libraries we received after the system was working.) Of the many cDNA isolates Chevray obtained (30), two turned out to encode Fos zipper-interacting proteins of interest to us based on their potential transcriptional effects: 1) FZA-A, a previously undescribed protein that stimulates Fos-dependent transcription in transfected cells and 2) FZA-B, a protein with 74% sequence identity to SUG1 of *S. cerevisiae* (31), later implicated in specific transcription *in vitro* by RNA polymerase II holoenzyme

(32). FZA-B and SUG1 have conserved ATP-binding domain sequences typical of ATPases and a region predicted to form coiled coil interactions with other proteins. We concentrated primarily on FZA-B. Subsequently, Hughes Associate Timothy Schaefer used Chevray's strains and vectors to isolate mammalian cDNAs encoding proteins that interact with the N-terminal region of c-Jun, which was known to have protein-binding regulatory sites (33,34) and to have only partial sequence similarity to JunB and JunD (35). By this means Schaefer identified several potentially interesting regulatory proteins (Table 1). We concentrated on one of the homeodomain proteins (Hex) and Stat3 β .

FZA-B/Fos (27). That FZA-B is the functional mammalian counterpart of SUG1 was shown by its ability to substitute for SUG1 in *S. cerevisiae* (27). (Similar results were reported by two other groups, who discovered mammalian Sug1 by its interaction in yeast with other transcription factors (35,36)). Hughes Associate Wenlan Wang then investigated whether FZA-B (hereafter called Sug1) is present in cells as part of a multiprotein mediator of transcription, as reported for yeast SUG1 (32). Fractionation of rat liver or HeLa cell extracts indicated that Sug1 was present in a high molecular mass complex that co-purified with 26S proteasomes. Only nuclear 26S proteasomes appeared to contain Sug1. Antibody against a Sug1-specific peptide depleted most of the proteasomal activity, proteasomal proteins and c-Fos from a proteasome preparation (27). Therefore Sug1 is an integral component of the nuclear 26S proteasome, which are already known to contain at least six putative ATPase subunits (64). SUG1 has also been found to be a component of 26S proteasomes in *S. cerevisiae* (38). These findings change the focus of work on Sug1 to its possible role in the degradation of transcription factors with which it interacts, as discussed below.

Jun/Hex. We were interested in the interaction of homeodomain proteins and Jun because the former are a large class of transcription factors implicated in development and because we found that Hex expression in transfected cells completely inhibits c-Jun or c-Jun/Fos-dependent transcription of a reporter gene. However, the same effect was observed with JunB and JunD, so Hex was not specific for c-Jun as we had anticipated. The homeodomain of Hex was essential for the transcriptional effect and Jun interaction, but the N-terminus of c-Jun was not required for either homeodomain interaction *in vitro* or the transcriptional effect of Hex (even though Hex was identified in the yeast screen by interaction with an N-terminal peptide of c-Jun). We now believe, but have not proven, that the relevant interaction is between the bZip of Jun and the homeodomain of Hex.

Stat3 β /Jun (26). Timothy Schaefer discovered Stat3 β by its interaction with c-Jun in the two-hybrid system. (Such an isoform of Stat3 had been suspected, since a short form of Stat3 had been repeatedly observed in cell extracts.) This interaction was of interest to us, because Stat proteins are latent DNA-binding transcription factors present in the cytoplasm that are very rapidly activated by growth factors and cytokines and move to the nucleus to activate specific immediate early genes (reviewed in 23). Six related Stat family members have been identified, Stat1 to 6, and for some of these a long (α) form and a short (β) form and other splice variants have been described. The functional domains of a typical Stat protein are shown in Figure 1. Of particular note are 1) the conserved tyrosine around residue 700, phosphorylation of which leads to

dimerization, nuclear import, and DNA binding activity; 2) an SH2 domain involved in dimerization and interaction with tyrosine protein kinases, 3) a possible SH3 domain, 4) DNA-binding domains (39,40), and a phosphorylatable serine residue near the C-terminus implicated in enhanced transcriptional activity (41). Cytokine-specific patterns of Stat activation, based on specific interactions between cytoplasmic domains of cytokine receptors and the SH2 domains of Stats, explain in part the specificity of biological activities of specific cytokines (41-43). Different Stat proteins bind optimally to similar, but not identical DNA sequences of the general type TTN₃-5AA, the length of the spacer being an important determinant of specificity (39,44). The transcriptional cooperativity of Stat3 β with Jun proteins (see below) is an illustration of a likely additional dimension of specificity of the genomic response to cytokines and growth factors based on cooperativity between particular Stat proteins and other transcription factors (see also 45).

Using the cDNA cloned via the yeast screen, Schaefer and Laura Sanders (Hughes Senior Research Technician) cloned and sequenced Stat3 β cDNA from mouse liver. From the sequence we inferred that Stat3 β is missing the 55 C-terminal amino acids of Stat3 α and has 7 unique amino acids at its C-terminus (26). This comes about presumably by RNA splicing that results in a deletion of 50 bp present in Stat3 α mRNA. In a comparison of Stat3 α and β activities (26,46), both isoforms were activated by the same set of cytokines and growth factors in Cos cells, and both formed DNA-binding homodimers as well as heterodimers with Stat1. Stat3 β differs functionally from Stat3 α in several respects, however. Unlike Stat3 α , which required growth factor or cytokine for activation of its DNA binding activity, Stat3 β was constitutively activated and constitutively phosphorylated on tyrosine 705. Stat3 β and c-Jun synergistically activated transcription from a promoter element containing both Stat and Jun binding sites, whereas Stat3 α showed only an additive effect with c-Jun. Based on these findings it is likely that Stat3 α and β have distinct cellular functions, and in particular activation distinct gene sets by differential interaction with other transcription factors.

Recently, Hughes Associate Ohkmae Park has found that purified recombinant Stat3 α and β (prepared in the baculovirus system with N-terminal His repeats) each formed a complex *in vitro* with the purified kinase domain of the EGF receptor prepared in insect cells (65). The kinase phosphorylated the Stats and activated them for DNA-binding (Figure 2). A Y705F mutation of Stat abolished DNA binding activity of the kinase product, and activation was blocked by a specific EGF-R kinase inhibitor. We believe this is the clearest demonstration to date that a growth factor receptor kinase can phosphorylate Y705 and activate Stats directly in the absence of associated proteins and is in agreement with other data pointing to the same conclusion (47,48).

Nup475 (49), also known as TIS11 (11) and tris-tetraprolin (12), is an immediate early zinc-binding nuclear protein of still unknown function that appears to have two novel Cys³His zinc fingers (10). Related proteins have been found in many eukaryotes. When Nup475 was identified, we suggested that it is a DNA-binding transcription factor, but we were not able to show specific DNA binding. We also were interested in the structure of the putative zinc fingers of Nup475. For metal binding and structural studies Hughes Associate Mark Worthington prepared peptides containing one or both copies

of the Cys3His repeats and a mutant form that had better solubility. In collaboration with Jeremy Berg's laboratory, these peptides were used to determine heavy metal binding by quantitating the characteristic spectroscopic transition seen in tetrahedrally coordinated Co^{2+} . By displacing Co^{2+} with Zn^{2+} , it was found that each Cys3His domain coordinates one Zn^{2+} (or Co^{2+}) ion, and the affinities are similar to those of typical zinc fingers (Figure 3). Worthington and Berg then determined the secondary structure of one of the metal binding domains by nuclear magnetic resonance spectroscopy (Figure 4). The structure is unique among metal-binding domains of proteins. Worthington (now at the University of Virginia) will continue working on the structure and function of Nup475.

HMG-I proteins (50). HMG-I genes (*HMG-I*, which gives rise to HMG-I and HMG-I(Y), and *HMG-I(C)* (51-53)) are activated by growth factors as part of the delayed early response (5). We therefore became interested in these DNA-binding, high mobility group chromosomal proteins from two points of view: transcriptional regulation of their genes by immediate early transcription factors, and function of the proteins. Dr. Linda Resar (a post-doctoral fellow in my laboratory and Assistant Professor of Pediatrics supported by an NIH First Award) examined transcriptional regulation of the HMG-I gene. Upstream of the major transcription start site she found a functional Myc/Max binding site. Deletion or point mutation of this site reduced gene expression by about two-thirds. Overexpression of Myc increased expression dependent on the site, and a dominant negative mutant of Myc inhibited serum-induced expression of HMG-I. Thus Myc appears to be an important activator of the HMG-I gene.

In regard to function, the HMG-I proteins are known to bind to AT tracts in the minor groove of DNA via three protein domains called AT hooks (54-56). HMG-I proteins have been shown to be involved in the formation of active transcription factor-DNA complexes ("enhanceosomes" (57-59). Hughes Associate Joseph Maher prepared purified recombinant HMG-I, HMG-I(Y) and HMG-I(C) in *E. coli* in order to analyze their DNA binding properties. Using duplex oligonucleotides with one, two or three AT tracts of 5 bp separated by a GC-rich sequence of variable length, he showed that high affinity sites consist of two or three AT tracts separated by 4 to 8 bp (50). Single AT tracts even up to 10bp showed weaker binding than appropriately spaced 5 bp tracts. Similar high affinity multivalent binding was demonstrated for AT tracts implicated in the regulation of the interferon β gene (57) and the JunB gene (17). Our results suggest that effective DNA-binding of HMG-I proteins requires multivalent binding by two or three AT hooks of a single molecular to 2 or 3 suitably spaced AT tracts (Figure 5). In the three dimensional structure of a nucleosome the DNA binding sites would presumably be appropriately spaced on the surface of the nucleosome.

FUTURE RESEARCH PLANS

I intend to narrow the focus of my laboratory's research. For the next year or so I expect to extend our recent findings suggesting that Sug1 may be involved in the intranuclear degradation of transcription factors. However, the main effort of the laboratory in the immediate future will be the further characterization of Stat3 β and related proteins, including their functional interactions with Jun and other transcription factors.

Function of Sug1. As a result of the finding that Sug1 is an integral component of nuclear 26S proteasomes, hypotheses about the function of Sug 1 have shifted from involvement in specific transcription to involvement in the degradation of transcription factors. Specifically we want to test the possibility, based on the demonstrated interactions of Sug 1 and Fos (and other transcriptions factors (36)) that Sug1 "captures" certain classes of transcription factors for degradation. It is conceivable that such a pathway is linked to the transcriptional activity of the transcription factors. To test for the functional interaction between Sug1 and Fos in the cell we plan to use mammalian Sug1 and c-Fos in *S. cerevisiae*, since we can then use genetic methods. First, we will determine whether c-Fos expressed in yeast strains that have only mammalian Sug1 is present in nuclear 26S proteasomes and is degraded via the 26S proteasomal pathway. Then we would attempt to make changes in the Sug1 leucine zipper that inactivate its interaction with c-Fos but leave the Sug1 active enough in *S. cerevisiae* to support growth. We then hope to make reciprocal changes in the c-Fos zipper that restore interaction with the mutant Sug1. With such mutants in hand, we would measure c-Fos in proteasomes and rates of c-Fos degradation in *S. cerevisiae* expressing various combinations of wild type or mutant Sug1 and c-Fos. Wenlan Wang has just begun experiments to obtain mammalian Sug1-expressing yeast strains and also to construct the Sug1 mutants with help from Jeff Boeke's laboratory. I anticipate that she will take this entire project with her when she leaves my laboratory.

Stat3 β . Unique properties of Stat 3 β and related Stat proteins will be the primary interest of my laboratory for the next few years. We plan to follow-up on several facets of Stat3 β function: 1) cooperativite interactions of Stat3 β with members of the Jun family, 2) possible cooperativity with other transcription factors, 3) the basis of quantitative differences between the DNA-binding activities of Stat3 α and β , 4) the interaction of Stat3 proteins with EGF receptor kinase, and 5) the possible role of constitutive activation of Stat3 β in neoplasia. In addition, I want to extend our findings on Stat3 β to other members of the Stat family. Specifically, we intend to determine: 6) whether other pairs of Stat α and β isoforms have differential properties similar to those of Stat3 α vs β . The results should contribute to understanding the biological specificities of cytokines and growth factors.

1) Cooperativity of Stat3 β and Jun proteins. Stat3 β , but not Stat3 α , acts cooperatively in transfected cells with Jun or Jun/Fos proteins in the activation of a promoter that has binding sites for both Stat3 and Jun. Such cooperativity is likely to be a basis for different transcriptional effects of the two isoforms. This differential effect of Stat3 β correlates with its interaction with the N-terminal segment of c-Jun in the yeast two hybrid assay. We want to determine whether cooperativity is evident at the DNA binding step and what features of the interacting proteins are essential for physical interaction and cooperative DNA binding.

Recent experiments on cooperative binding of c-Jun and Stat3 β to an α -2-macroglobulin promoter segment used in earlier experiments indicate that there is cooperative binding to this segment of DNA as assessed by footprinting (Figure 6). Stat3 α does not show cooperative binding with c-Jun. Using a gel shift assay, we plan to quantitate the cooperative effect by measuring the rate of release of bound Stat or Jun in the presence of excess binding site for the other protein, comparing the release of a protein in the presence or absence of a saturating concentration of the other protein. We also plan to examine various altered forms of Stat3 α and β to determine the structural basis for their different activities with Jun. Although there is a correlation between cooperativity with Jun of Stat3 α and β and interaction with the N-terminal segment of c-Jun in the two hybrid system, it is not clear that this physical interaction is the basis of cooperativity. In preliminary experiments Hughes Associate Ohkmae Park has found that interaction of Stat3 β and Jun *in vitro* is independent of the N-terminal region of Jun but depends on the bZip domain. We intend to examine various constructs of Jun and Stat3 for both *in vitro* interaction and cooperativity to define better the structural basis of the cooperative effect. Finally, we will determine whether natural Stat3-responsive promoters (other than the α -2-macroglobulin promoter) are cooperatively controlled by Stat3 β and Jun.

2) Possible cooperativity between Stat3 β and other transcription factors. To determine whether transcriptional cooperativity of Stat3 β extends beyond the Jun family, we plan to test other bZip transcription factors as well as other structural classes of transcription factors for cooperativity, initially by assays in transfected cells using artificial promoters with suitable protein-binding sites, and natural promoters with such sites. Since Stat3 β mRNA is present in many tissues, such cooperative effects could be widespread.

3) Basis of the quantitative differences in activities of Stat3 α and β . Associate Ohkmae Park has recently purified recombinant Stat3 α and β from insect cells expressing mammalian JAK1 or JAK2. The proteins are tyrosine phosphorylated and active for DNA binding; however, the ratio of p-tyr to protein for Stat3 β was approximately 3 to 6 times that for Stat3 α . Moreover, phosphorylated Stat3 β was approximately 30 to 60-fold more active than Stat3 α on a molar basis. Our working hypothesis is that Stat3 β is a better substrate for JAK1 and JAK2, and that Stat3 β dimers (the active form) are more stable than Stat3 α dimers. Recently Timothy Schaefer has obtained preliminary indication that activated Stat3 β is more stable than Stat3 α in EGF-stimulated Cos cells expressing Stat3 transgenes.

To determine the stability of activated cellular Stat3 isoforms more reliably, we plan to measure the decay of DNA binding activities of Stat3 α and Stat3 β in

transfected cells after stimulating activation of the Stat proteins with EGF and then preventing additional activation by adding a specific inhibitor of the EGF receptor kinase, which has been shown to inhibit Stat3 activation by EGF (46). We will also measure the rate of loss of phosphotyrosine in Stat proteins and the rate of loss of the proteins after inhibition of protein synthesis by using a monoclonal antibody that reacts with both Stat3 isoforms. This will help to determine whether decay of DNA-binding activity is due to dephosphorylation via a protein phosphatase, or proteolytic degradation. In addition to the cell experiments, we also plan to compare the stabilities of dimers of purified Stat3 α and β *in vitro* to determine whether any differences observed in cells are intrinsic to the proteins rather than due to extrinsic factors like susceptibility to protein phosphatases. (Since the quantitative differences in DNA-binding activities of phosphorylated Stat3 α and β noted above have recently been observed by Ohkmae Park with Stat3 preparations phosphorylated *in vitro* by EGF receptor kinase, we believe these differences are largely due to intrinsic properties of the activated proteins.) If the early results are positive, we would extend these studies by determining what structural features of Stat3 β are required for increased stability and by testing the stabilities of various Stat3 homodimers and heterodimers prepared from purified proteins. Stability of various Stat dimers could be another important determinant of the genomic response to different cell surface ligands that exert their effects through Stat proteins.

4) Interaction of Stat3 and EGF receptor kinase. As previously noted, Ohkmae Park found that EGF receptor kinase activates Stat3 proteins *in vitro*. She also observed that a stable complex forms between the phosphorylated forms of Stat and the receptor, requiring ionic detergent for dissociation. We plan to use this *in vitro* system to study the reaction in more detail, e.g., to determine the smallest segment of Stat that interacts and the effects of mutations around Y705 and in the SH2 and SH3 domains of Stat; the effects of added purified JAK; and using the dissociation of the receptor-Stat complex as an assay, what is required to release phosphorylated Stat from the receptor.

5) Possible role of constitutive activation of Stat3 β in neoplasia. Oncoproteins that are constitutively active forms of growth factor receptors (e.g., mutant GM-CSF, and EGF and Neu receptors) have long been known, as have altered forms of transcription factors that are induced by growth factors. Therefore it is a reasonable hypothesis that constitutively active forms of certain Stat proteins may also be oncogenic if overproduced. We therefore plan to determine whether high level expression of Stat3 β occurs in neoplastic cells, and whether Stat3 β transforms cells either alone or together with other proteins.. At least two instances of elevated Stat 3 activity (isoform not determined) have been reported, namely high constitutive activity of Stat 3 in cells transformed by src (60) and in HTLV-infected cells (61). Schaefer recently found that Stat3 β (detectable with 3 β -specific antibody) appeared to be elevated in a number of human leukemia cell lines. Schaefer is now examining a series of other tumor cells for Stat3 β overexpression and activity. If there is a good correlation between Stat3 β levels and specific neoplasms or if Stat transforms cells in culture, he will attempt to test the relevance of high levels of Stat3 β to the growth phenotype by attempting to alter the growth phenotype with

a dominant negative form of Stat3 β (62). Schaefer plans to take this project with him when he leaves my laboratory.

6) Differential properties of other Stat isoforms. Other Stat proteins are present in long (α) and short (β) forms, as first described for Stat1. We would like to see whether some of the short forms have properties like those of Stat3 β (constitutively active, cooperative interactions with other transcription factors, possibly form more stable dimers) by doing experiments like those already described for Stat3 β .

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